## Research Note

# Efficacy of Iron Chelators on *Campylobacter* Concentrations in Turkey Semen[1]

K. Cole,* A. M. Donoghue,† I. Reyes-Herrera,* N. Rath,† and D. J. Donoghue*[2]

*Department of Poultry Science, University of Arkansas, Fayetteville 72701; and †Poultry Production and Product Safety Research Unit, ARS, USDA, Fayetteville, AR 72701

**ABSTRACT** *Campylobacter* is a leading bacterial cause of human foodborne infections in the United States. Recent studies suggest that the organism is highly prevalent in poultry semen and may contribute to vertical transmission between the breeder hen and offspring. Because *Campylobacter* requires iron for its growth and survival, the objective of this study was to determine if the addition of natural and synthetic chelators such as ovotransferrin, desferrioxaime, EDTA, or 2,2′-dipyridyl could reduce or eliminate *Campylobacter* in turkey semen. In a preliminary study without semen, a commercial poultry semen extender was supplemented with various concentrations of ovotransferrin, desferrioxaime, EDTA, or 2,2′-dipyridyl and inoculated with an average of $10^8$ cfu/mL of a wild-type *Campylobacter coli* turkey semen isolate. At 6 and 24 h of storage at 4°C, a sample was taken from each treatment group and enumerated for *Campylobacter.* In all 3 trials, *Campylobacter* was undetectable ($<10^2$) in the commercial poultry semen extender supplemented with 20 mg/mL of 2,2′-dipyridyl. There were no differences observed in *Campylobacter* concentrations in the commercial poultry semen extender supplemented with ovotransferrin, desferrioxaime, or EDTA compared with unsupplemented controls. In a follow-up study, pooled semen samples were randomly collected from toms, diluted with a commercial poultry semen extender supplemented with 5, 10, or 20 mg/mL of 2,2′-dipyridyl and inoculated with an average of $10^8$ cfu/mL of a wild-type *C. coli* turkey semen isolate. At 6 and 24 h of storage at 4°C, samples were taken from each treatment group, enumerated for *Campylobacter,* and evaluated for sperm viability. In all 3 trials, supplementing the commercial poultry semen extender with 20 mg/mL of 2,2′-dipyryidyl significantly reduced (3 to 4 logs) *Campylobacter* concentrations when compared with the positive controls. Sperm viability was also reduced with this treatment, and, therefore, the use of 2,2′-dipyridyl may not be a practical treatment for reducing *Campylobacter* in poultry semen.

**Key words**: *Campylobacter,* iron, turkey, semen

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## INTRODUCTION

*Campylobacter* is one of the leading bacterial causes of human foodborne infections in the United States (Friedman et al., 2000; Centers for Disease Control and Prevention, 2005). Epidemiological evidence has emphasized the importance of poultry products as a significant source of human *Campylobacter* infection (Jacobs-Reitsma, 2000; Corry and Attabay, 2001). Recent studies suggest that the organism is highly prevalent in turkey semen and may contribute to vertical transmission between the breeder hen and offspring (Cox et al., 2002; Cole et al., 2004a,b). Semen on commercial turkey farms is routinely pooled and used to inseminate multiple hens, and, therefore, may be a potential source of *Campylobacter* contamination in the female reproductive tract and subsequent eggs. Unfortunately, strategies to reduce or eliminate *Campylobacter* in poultry semen, such as aeration, reduced storage temperatures, and dilution with extenders containing antibiotics have not been completely effective (Cole et al., 2004b; Donoghue et al., 2004).

*Campylobacter,* like most organisms, requires iron for its growth and survival (van Vliet et al., 2002; Palyada et al., 2004). Numerous in vitro studies have demonstrated that limiting the availability of iron in the environment can inhibit certain strains of *Escherichia coli* (Chart and Rowe, 1993), *Salmonella* (Chart and Rowe, 1993; Lisiecki et al., 2000; Ho et al., 2004), and *Campylobacter* (Field et al., 1986; Holmes et al., 2005). Limiting iron can be accomplished by the addition of natural or synthetic chelators such as ovotransferrin, desferrioxaime, EDTA, and 2,2′-dipyridyl to the growth media (Chart and Rowe, 1993;


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[2]Corresponding author: ddonogh@uark.edu

**Table 1.** Effects of 2,2′-dipyridyl on percentage motility of turkey spermatozoa stored in vitro for 6 or 24 h[1]

| | Trial 1 | | Trial 2 | | Trial 3 | |
|---|---|---|---|---|---|---|
| Treatment (%) | 6 h | 24 h | 6 h | 24 h | 6 h | 24 h |
| Positive control | 80[a] | 70[a] | 90[a] | 70[a] | 80[a] | 70[a] |
| Negative control | 80[a] | 70[a] | 90[a] | 70[a] | 80[a] | 70[a] |
| 5 mg/mL of 2,2′-dipyridyl | 70[b] | 50[b] | 70[b] | 50[b] | 70[b] | 50[b] |
| 10 mg/mL of 2,2′-dipyridyl | 50[c] | 10[c] | 50[c] | 10[a] | 50[c] | 10[c] |
| 20 mg/mL of 2,2′-dipryidyl | 40[d] | 0[d] | 40[d] | 0[d] | 30[d] | 0[d] |

[a–d]Means with no common superscript within columns differ significantly ($P < 0.05$).

[1]In 3 separate trials, semen samples from commercial toms were randomly collected by abdominal massage, aspirated into sterile test tubes, and pooled. The pooled semen samples were diluted 1:4 (vol:vol) with a commercial poultry semen extender and supplemented with 5, 10, or 20 mg/mL of 2,2′-dipyridyl. Treatment groups inoculated with *Campylobacter* were inoculated with 0.25 mL of *Campylobacter* enrichment broth containing $10^8$ cfu/mL of a wild-type *Campylobacter coli* turkey semen isolate. Each treatment was incubated at 4°C for 24 h with agitation (150 rpm). Sperm motility was assessed for each treatment group at 6 and 24 h of storage using the hanging drop method.

Bergan et al., 2001; Ho et al., 2004). Theoretically, supplementing semen diluents with these iron-chelating agents should reduce or eliminate *Campylobacter* concentrations in poultry semen. In addition, desferrioxaime has been used to improve sperm viability during in vitro storage in some species (Vishwanath et al., 1994). Therefore, the objective of the present study was to determine whether supplementation with natural and synthetic chelators could reduce *Campylobacter* concentrations in pooled turkey semen.

## MATERIALS AND METHODS

In preliminary dose titration studies conducted without semen, 0.25 mL of *Campylobacter* enrichment broth (**CEB**) containing an average of $10^8$ cfu/mL of a wild-type *Campylobacter coli* semen isolate was added to 1.75 mL of Field Ready Green Extender (no antibiotics; IMV International Corp., Maple Grove, MN) supplemented with either 0.5, 5, or 50 mg/mL of ovotransferrin (Sigma Chemical Co., St. Louis, MO); 0.1, 1, or 10 mg/mL of desferrioxaime (Desferal, Ciba-Geigy Corp. Ltd., Basel, Switzerland); 0.1, 1, or 10 mg/mL of EDTA (Sigma Chemical Co.); or 0.2, 2, or 20 mg/mL of 2,2′-dipyridyl (Sigma Chemical Co.) in 3 separate trials. *Campylobacter* concentrations ranging from $10^2$ to $10^6$ cfu/mL in turkey semen have been previously reported (Cole et al., 2004a); however, we have found levels as high as $10^8$ cfu/mL in turkey semen samples since that report. We chose this higher concentration for challenge because we believed that if we could demonstrate efficacy at the highest levels of *Campylobacter* in semen this would also be effective at lower concentrations. In the present studies, 1.75 mL of Field Ready Green Extender (IMV International Corp.) inoculated with 0.25 mL of CEB containing an average of $10^8$ cfu/mL of a wild-type *C. coli* semen isolate served as the positive controls, whereas 1.75 mL of Field Ready Green Extender (IMV International Corp.) inoculated with 0.25 mL of CEB alone served as the negative controls. Each treatment was incubated at 4°C for 24 h with agitation (150 rpm; Thurston et al., 1998). At 6 and 24 h, a 0.1-mL sample was taken from each treatment group and diluted with 0.9 mL of CEB, 10-fold serial dilutions in CEB were performed, and 0.1 mL of each dilution was plated on Campy-Line agar (**CLA**; Line, 2001) and incubated at 42°C for 48 h in a microaerophilic environment (5% $O_2$, 10% $CO_2$, 85% $N_2$). After incubation, characteristic colonies were confirmed as *Campylobacter* by observation of typical cellular morphology using a phase contrast microscope and with a commercial latex agglutination test kit (Pan Bio Inc., Columbia, MD) specific for *Campylobacter jejuni, C. coli,* and *Campylobacter laridis.* The colonies on each CLA plate were counted on a Leico Darkfield plate colony counter (Leica Inc., Buffalo, NY), and the direct counts were converted to $\log_{10}$ colony-forming units per milliliter of extender.

In a follow-up study, pooled semen samples from commercial toms were randomly collected by abdominal massage (Burrows and Quinn, 1937) and aspirated into sterile test tubes. In 3 separate trials, the pooled semen samples were diluted 1:4 (vol:vol) with Field Ready Green Extender (no antibiotics; IMV International Corp.) and divided into 5 treatment groups. The positive and negative control groups received no 2,2′-dipyridyl, whereas the remaining 3 treatment groups were supplemented with either 5, 10, or 20 mg/mL of 2,2′-dipyridyl. Each treatment group, except for the negative control, was then inoculated with 0.25 mL of CEB containing an average of $10^8$ cells/mL of a wild-type *C. coli* semen isolate. Each treatment was incubated at 4°C for 24 h with agitation (150 rpm; Thurston et al., 1998). At 6 and 24 h of storage, a 0.1-mL sample was taken from each treatment group and serially diluted with CEB. The dilutions were plated on CLA and evaluated for *Campylobacter* as previously described. Sperm motility was assessed for each treatment group at 6 and 24 h of storage according to the hanging drop method of Wishart and Wilson (1997).

Data were analyzed by ANOVA using the Statistical Analysis System (SAS Institute Inc., 1998) GLM program. The numbers of *Campylobacter* colonies were logarithmically transformed ($\log_{10}$ cfu/mL) prior to analysis to achieve homogeneity of variance (Byrd et al., 2003; Cole et al., 2004b; Farnell et al., 2005). Treatment means were partitioned by LSMEANS analysis (SAS Institute Inc.,

**Table 2.** Efficacy of iron chelators against *Campylobacter* (cfu/mL) in poultry semen extender[1,2]

| | Trial 1 | | Trial 2 | | Trial 3 | |
|---|---|---|---|---|---|---|
| Treatment | 6 h | 24 h | 6 h | 24 h | 6 h | 24 h |
| Positive control | $1.7 \times 10^{7,a}$ | $1.0 \times 10^{7,a}$ | $6.6 \times 10^{6,a}$ | $1.7 \times 10^{7,a}$ | $9.8 \times 10^{6,a}$ | $2.0 \times 10^{7,a}$ |
| Negative control | $<10^{2,b}$ | $<10^{2,b}$ | $<10^{2,b}$ | $<10^{2,b}$ | $<10^{2,b}$ | $<10^{2,b}$ |
| 0.5 mg/mL of ovotransferrin | $1.7 \times 10^{7,a}$ | $1.1 \times 10^{7,a}$ | $1.5 \times 10^{7,a}$ | $1.2 \times 10^{7,a}$ | $7.6 \times 10^{6,a}$ | $8.1 \times 10^{6,a}$ |
| 5 mg/mL of ovotransferrin | $1.8 \times 10^{7,a}$ | $1.6 \times 10^{7,a}$ | $1.1 \times 10^{7,a}$ | $1.4 \times 10^{7,a}$ | $6.3 \times 10^{6,a}$ | $1.0 \times 10^{7,a}$ |
| 50 mg/mL of ovotransferrin | $1.5 \times 10^{7,a}$ | $1.4 \times 10^{7,a}$ | $1.4 \times 10^{7,a}$ | $1.3 \times 10^{7,a}$ | $8.2 \times 10^{6,a}$ | $9.3 \times 10^{6,a}$ |
| 0.1 mg/mL of desferrioxaime | $9.1 \times 10^{6,a}$ | $6.4 \times 10^{6,a}$ | $1.8 \times 10^{7,a}$ | $1.8 \times 10^{7,a}$ | $1.2 \times 10^{7,a}$ | $8.5 \times 10^{6,a}$ |
| 1 mg/mL of desferrioxaime | $1.2 \times 10^{7,a}$ | $6.8 \times 10^{6,a}$ | $1.4 \times 10^{7,a}$ | $1.7 \times 10^{7,a}$ | $1.0 \times 10^{7,a}$ | $1.2 \times 10^{7,a}$ |
| 10 mg/mL of desferrioxaime | $1.4 \times 10^{7,a}$ | $9.8 \times 10^{6,a}$ | $6.4 \times 10^{6,a}$ | $9.0 \times 10^{6,a}$ | $9.7 \times 10^{6,a}$ | $9.0 \times 10^{6,a}$ |
| 0.1 mg/mL of EDTA | $1.1 \times 10^{7,a}$ | $1.6 \times 10^{6,a}$ | $5.7 \times 10^{6,a}$ | $8.7 \times 10^{6,a}$ | $1.2 \times 10^{7,a}$ | $7.5 \times 10^{6,a}$ |
| 1 mg/mL of EDTA | $9.0 \times 10^{6,a}$ | $1.6 \times 10^{6,a}$ | $7.1 \times 10^{6,a}$ | $1.0 \times 10^{7,a}$ | $7.1 \times 10^{6,a}$ | $1.1 \times 10^{7,a}$ |
| 10 mg/mL of EDTA | $1.1 \times 10^{7,a}$ | $6.5 \times 10^{5,a}$ | $8.4 \times 10^{6,a}$ | $5.1 \times 10^{6,a}$ | $1.0 \times 10^{7,a}$ | $7.8 \times 10^{6,a}$ |
| 0.2 mg/mL of 2,2′-dipyridyl | $1.9 \times 10^{6,a}$ | $7.4 \times 10^{5,a}$ | $1.9 \times 10^{6,a}$ | $8.0 \times 10^{6,a}$ | $9.2 \times 10^{6,a}$ | $5.2 \times 10^{5,a}$ |
| 2 mg/mL of 2,2′-dipyridyl | $9.7 \times 10^{5,a}$ | $1.5 \times 10^{6,a}$ | $1.4 \times 10^{6,a}$ | $1.1 \times 10^{6,a}$ | $8.6 \times 10^{6,a}$ | $6.6 \times 10^{5,a}$ |
| 20 mg/mL of 2,2′-dipyridyl | $<10^{2,b}$ | $<10^{2,b}$ | $<10^{2,b}$ | $<10^{2,b}$ | $<10^{2,b}$ | $<10^{2,b}$ |

[a,b]Means with no common superscript within columns differ significantly ($P < 0.05$).

[1]All data were $\log_{10}$ transformed for statistical analysis. For clarity of presentation, arithmetic means are presented.

[2]In 3 separate trials, 0.25 mL of *Campylobacter* enrichment broth containing an average $10^8$ cfu/mL of a wild-type *Campylobacter coli* turkey semen isolate was added to 1.75 mL of a commercial poultry semen extender supplemented with various concentrations of ovotransferrin, desferrioxaime, EDTA, or 2,2′-dipyridyl. Each treatment group was incubated at 4°C for 24 h with agitation (150 rpm).

1998). Sperm motility data expressed as percentages (Table 1) were arc sine transformed before analysis. A probability of $P < 0.05$ was required for statistical significance. The data in Tables 2 and 3 are shown as arithmetic means for clarity of presentation.

## RESULTS AND DISCUSSION

Preliminary studies were conducted without semen to determine if natural or synthetic chelators could effectively reduce *Campylobacter* in a commercial poultry semen extender. In these studies, there were no differences observed in *Campylobacter* concentrations in the commercial poultry semen extender supplemented with either 0, 0.5, 5, or 50 mg/mL of ovotransferrin; 0, 0.1, 1, or 10 mg/mL of desferrioxaime; 0, 0.1, 1, or 10 mg/mL of EDTA; or 0, 0.2, or 2 mg/mL of 2,2′-dipyridyl (Table 2). However, in all 3 trials, *Campylobacter* levels were undetectable ($<10^2$) at 6 or 24 h of storage in commercial poultry semen extender supplemented with 20 mg/mL of 2,2′-dipyridyl compared with the positive controls ($P < 0.05$). The reduction of *Campylobacter* in the presence of 20 mg/mL of 2,2′-dipyridyl but not in the presence of 0.2 or 2 mg/mL of 2,2′-dipyridyl suggests a dose-dependent response.

In a follow-up study, pooled turkey semen was diluted with a commercial poultry semen extender supplemented with 5, 10, or 20 mg/mL of 2,2′-dipyridyl. Similar to the results in the previous study, the only reduction in *Campylobacter* concentrations was observed at 6 or 24 h of storage in commercial poultry semen and extender supplemented with 20 mg/mL of 2, 2′-dipyridyl (Table 3). However, the presence of 2,2′-dipyridyl adversely affected sperm motility (Table 1).

The mechanism by which 2,2′-dipyridyl reduced *Campylobacter* concentrations in these studies is unclear. It has been reported that 2,2′-dipyridyl can cause lysis

**Table 3.** Efficacy of 2,2′-dipyridyl against *Campylobacter* (cfu/mL) in pooled turkey semen stored in vitro for 6 or 24 h[1,2]

| | Trial 1 | | Trial 2 | | Trial 3 | |
|---|---|---|---|---|---|---|
| Treatment | 6 h | 24 h | 6 h | 24 h | 6 h | 24 h |
| Positive control | $2.8 \times 10^{6,a}$ | $2.0 \times 10^{7,a}$ | $8.7 \times 10^{6,a}$ | $8.1 \times 10^{6,a}$ | $7.8 \times 10^{6}$ | $9.8 \times 10^{6,a}$ |
| Negative control | $<10^{2,b}$ | $<10^{2,b}$ | $<10^{2,b}$ | $<10^{2,b}$ | $<10^{2,b}$ | $<10^{2,b}$ |
| 5 mg/mL of 2,2′-dipyridyl | $5.8 \times 10^{6,a}$ | $1.1 \times 10^{7,a}$ | $3.1 \times 10^{6,a}$ | $3.9 \times 10^{6,a}$ | $4.8 \times 10^{6,a}$ | $6.5 \times 10^{6,a}$ |
| 10 mg/mL of 2,2′-dipyridyl | $5.2 \times 10^{6,a}$ | $4.7 \times 10^{6,a}$ | $7.1 \times 10^{6,a}$ | $1.5 \times 10^{6,a}$ | $9.2 \times 10^{6,a}$ | $1.2 \times 10^{6,a}$ |
| 20 mg/mL of 2,2′-dipyridyl | $1.6 \times 10^{3,b}$ | $2.2 \times 10^{3,b}$ | $2.2 \times 10^{3,b}$ | $2.1 \times 10^{3,b}$ | $2.0 \times 10^{2,b}$ | $<10^{2,b}$ |

[a,b]Means with no common superscript within columns differ significantly ($P < 0.05$).

[1]All data were $\log_{10}$ transformed for statistical analysis. For clarity of presentation, arithmetic means are presented.

[2]In 3 separate trials, semen samples from commercial toms were randomly collected by abdominal massage, aspirated into sterile test tubes, and pooled. The pooled semen samples were diluted 1:4 (vol:vol) with a commercial poultry semen extender and supplemented with 5, 10, or 20 mg/mL of 2,2′-dipyridyl. Each treatment group was then inoculated with 0.25 mL of *Campylobacter* enrichment broth containing $10^8$ cfu/mL of a wild-type *Campylobacter coli* turkey semen isolate. Each treatment was incubated at 4°C for 24 h with agitation (150 rpm).

in bacterial cells (Neilands, 1982; Chart et al., 1986). In addition, 2,2′-dipyridyl is able to bind cellular iron effectively, producing reactive oxygen species that lead to apoptosis in cancer cells (Yuan et al., 2004). Although reactive oxygen species production was not measured in the semen in this study, this may be a possible explanation for the bactericidal effects observed in this study. The production of free radicals may also explain the spermicidal effects observed in this study, as they can cause irreversible damage in sperm membranes (Wishart, 1984; Ravie and Lake, 1985).

In conclusion, *Campylobacter* concentrations were significantly reduced after 6 or 24 h of storage at 4°C in a commercial poultry semen extender supplemented with 20 mg/mL of 2,2′-dipyridyl. This approach, however, is not a practical solution to reduce *Campylobacter* concentrations in semen, because this treatment also reduced sperm motility. Further studies are needed to find a practical means of reducing or eliminating pathogens in poultry semen without adversely affecting sperm viability and subsequent function.

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